Development of new tools for molecular diagnosis of hematologic malignancies

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ABSTRACT

Molecular alterations represent diagnostic and prognostic factors in hematologic diseases. Their presence can anticipate the malignancy outcome or confer responsiveness to specific treatments. Here we present the development of new assays based on an innovative technology, named loop-mediated isothermal amplification (LAMP), for the molecular detection of these alterations in a simple, fast and accurate way. These assays can identify molecular aberrations in a non polymerase chain reaction-based manner, starting directly from DNA or RNA in a single-tube close format and in a short time frame.

INTRODUCTION

After the initial morphologic evaluation of blood cells, the diagnostic work-up of hematologic malignancies contemplates the detection of molecular markers essential for diagnosis confirmation and for risk stratification. These data drive the therapy choice and impact on the patient management and outcome. Currently, the identification of molecular alterations involved in hematologic diseases is performed by methods based on polymerase chain reaction (PCR) system and Sanger sequencing. Upcoming new technologies, such as next generation sequencing (NGS), could represent powerful tools to study large, selected genomic regions spanning a huge amount of genetic alterations (1). Although these technologies allow to have a lot of molecular information in a relatively short time frame, the presence of some specific markers should be known in a few days, even in few hours, to define the diagnosis and promptly start treatments. For example, in case of suspected acute promyelocytic leukemia (APL), the accuracy and speed of the diagnostic work-up, which include the identification of *PML-RARA* chimeric transcripts, is mandatory to start the therapy with all-trans retinoic acid (ATRA) combined with anthracycline or arsenic trioxide (ATO). These therapeutic strategies have dramatically changed the natural history of APL, converting it from a fatal into a highly curable leukemia (2). Unfortunately, a significant proportion of patients still do not benefit from these effective treatments due to a delay of diagnosis that may lead to a life-threatening coagulopathy. Based on current guidelines of the LeukemiaNet panel, immediate recommended actions should be taken based on the sole morphologic suspicion of APL, but confirmation of diagnosis at the genetic level is considered essential for patient eligibility to ATRA and/or ATO-based treatments (3). A similar scenario arise at presentation of chronic myeloid leukemia (CML) and B precursor acute lymphoblastic leukemia (Bp-ALL), in which the molecular detection of *BCR-ABL1* transcripts, arising from the t(9;22) translocation, is routinely performed to confirm diagnosis for the former and to define the risk class for the latter. Patients positive for this molecular marker can benefit of a targeted therapy, represented by the use of inhibitors of ABL1-tyrosine kinase (TKI). In CML, the use of single agent TKI currently represent the first line therapy (4), whereas in ALL they can be administrated either alone or in combination with chemotherapy (5-8). The usage of TKI led to an extraordinary improvement of survival in both these types of leukemias (7, 9). Other clinical and biologic entities belonging to the chronic

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myeloproliferative neoplasm (MPN) family encompass polycythemia vera (PV), essential thrombocytemia (ET) and myelofibrosis (MF), all together known as Philadelphia-negative MPN. A common point mutation within the *JAK2* gene is detected in almost all PV and half of ET and MF patients. This mutation causes an amino acid shift (JAK2 V617F) in the coded protein and the mutated vs. wild type allelic ratio can vary in positive patients from 100% to 1%, conferring a different disease outcome (10). The identification of this point mutation is also considered essential during the diagnostic work-up of a myeloproliferative neoplasm by WHO classification (11).

Robust and reliable molecular tests have been developed during the last decades for all afore mentioned molecular markers. Most of these methods are based on qualitative (12, 13) or quantitative PCR (RQ-PCR) (14, 15), and new technologies (as digital PCR) are also facing the hematologic diagnostic process (16, 17). Because of the high, intrinsic contamination proneness of the PCR-based technologies and the low automation level of these methods, these tests are usually performed by well trained operators in specialized laboratories. Results are clinically available in a period of time ranging from 4-5 days (depending on the laboratory organization) to not less than 4-5 h in case of the urgent *PML-RARA* determination. New rapid and simple technologies could give patients the opportunity to have a reliable molecular diagnosis also in small hospitals. This would ensure them prompt referral to specialized centers for proper treatments, including enrolment into clinical trials that can offer the access to innovative molecules. The loop-mediated isothermal amplification (LAMP) technology is a rapid and simple non PCR-based methodology already used in microbial and virus identification and quantification (18). More recently, we started employing this methodology in the hematologic field (19). In this report, we describe further development steps of LAMP-based assays for hematologic disease.

MATERIAL AND METHODS

Samples

We analyzed nucleic acids (RNA or DNA) extracted from bone marrow or peripheral blood cells of patients affected by APL, CML, ALL or MPN and from healthy donors. Total RNA was extracted by RNeasy mini kit (Qiagen) from guanidinium iso-thiocyanate (GITC) lysates of whole blood cells (WBC), mononuclear cells (MNC), granulocytes isolated by Ficoll-Hypaque gradient or cell lines. Genomic DNA was extracted by Gentra kit (Qiagen) from WBC or cell lines. This study was performed in accordance with the Declaration of Helsinki and informed consent was obtained from involved patients.

Cell lines

The following human cell lines were used to assess specificity and sensitivity of molecular LAMP procedures: NB4 [a t(15;17)-positive APL], HL60 (AML, bearing the BCOR mutation) (20), KASUMI-1 [a t(8;21) AML1/ETO-positive AML], K562 [a t(9;22) BCR/ABL1 p210-positive CML in erythroid blast crisis], TOM-1 [a t(9;22) BCR/ABL1 p190-positive ALL], 697 [a t(1;19) E2A/PBX1-positive ALL], RS411, MV4-11 [both t(4;11) MLL/AF4-positive ALL], REH [a t(12;21) TEL/AML1-positive ALL], B-JAB (a Burkitt non-Hodgkin's lymphoma) and UKE-1 (harboring JAK2 V617F mutation, a gift of Walter Fiedler, Eppendorf Hospital, Hamburg, Germany).

Conventional identification of chimeric transcripts by reverse transcriptase-PCR

Identification of *PML-RARA* and *BCR-ABL1* chimeric transcripts was performed on diagnostic total RNA by the two-step reverse transcriptase (RT)-PCR as previously described (12, 14). The RNA integrity and the efficiency of the retro-transcription step were evaluated for each cDNA by the amplification of the wild type ABL1 gene in a separate PCR reaction, as indicated by BIOMED recommendations (12, 14).

Conventional identification of JAK2 V617F mutation

The allele specific oligonucleotide (ASO)-PCR for the JAK2 V617F mutation was performed as previously described (21). Confirmation of positivity and estimation of allele burden was performed by amplification of exon 14 and subsequent digestion with BsaXI (New England Biolabs) (13).

LAMP assays

The LAMP reaction is a non-PCR isothermal method for rapid amplification of nucleic acids. This reaction consists in 4 primers specifically designed to recognize 6 distinct regions on the target gene: a pair of outer primers (F3 and B3) and a pair of inner primers (FIP and BIP) presenting a tag complementary to a downstream region in the opposite strand of the target (F1 and B1) (Figure 1). The LAMP reaction is performed at constant temperature thanks to the use of a polymerase with a strand displacement activity. The FIP and BIP primers anneal and are extended on the target and the newly synthesized chains are then displaced by extension of F3 and B3. The displaced product generates a "stem-loop" structure that represents the starting structure for a classical LAMP reaction (see http://www.haematologica.org/content/suppl/2013/08/02/haematol.2011.056184.DC2).

The basic LAMP assay was modified and implemented to identify hematologic molecular targets as detailed below.

For *PML-RARA* and *BCR-ABL1* transcripts amplification, the basic LAMP method has been modified by introducing a new polymerase with both RNA retrotranscription and DNA amplification activity, allowing

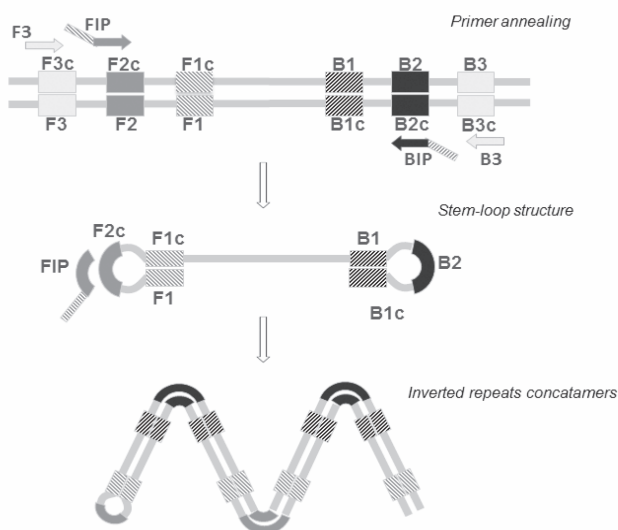


Figure 1
Scheme of the loop-mediated isothermal amplification (LAMP) method.
Two inner primers FIP and BIP are extended on the target DNA in isothermal conditions. The newly synthesized DNA chains are then extended by second two oligonucleotides F3 and B3. The LAMP reaction begins when the end of the single filament bend on itself creating a stem-loop structure. This displaced product is again annealed by the primers and extended by the polymerase generating an inverted repeats concatamers.

Table 1
Interpretation scheme for the correct discrimination of PML-RARA isoforms[a]

Detection bcr1,3 assay		Discrimination bcr2 assay	Sample Results
Channel bcr1 530 nm	Channel bcr3 570 nm	Channel bcr2 500 nm	
+	-	-	Bcr1
+	-	+	Bcr1
-	+	-	Bcr3
-	+	+	Bcr2
-	-	+	Bcr2
-	-	-	Negative

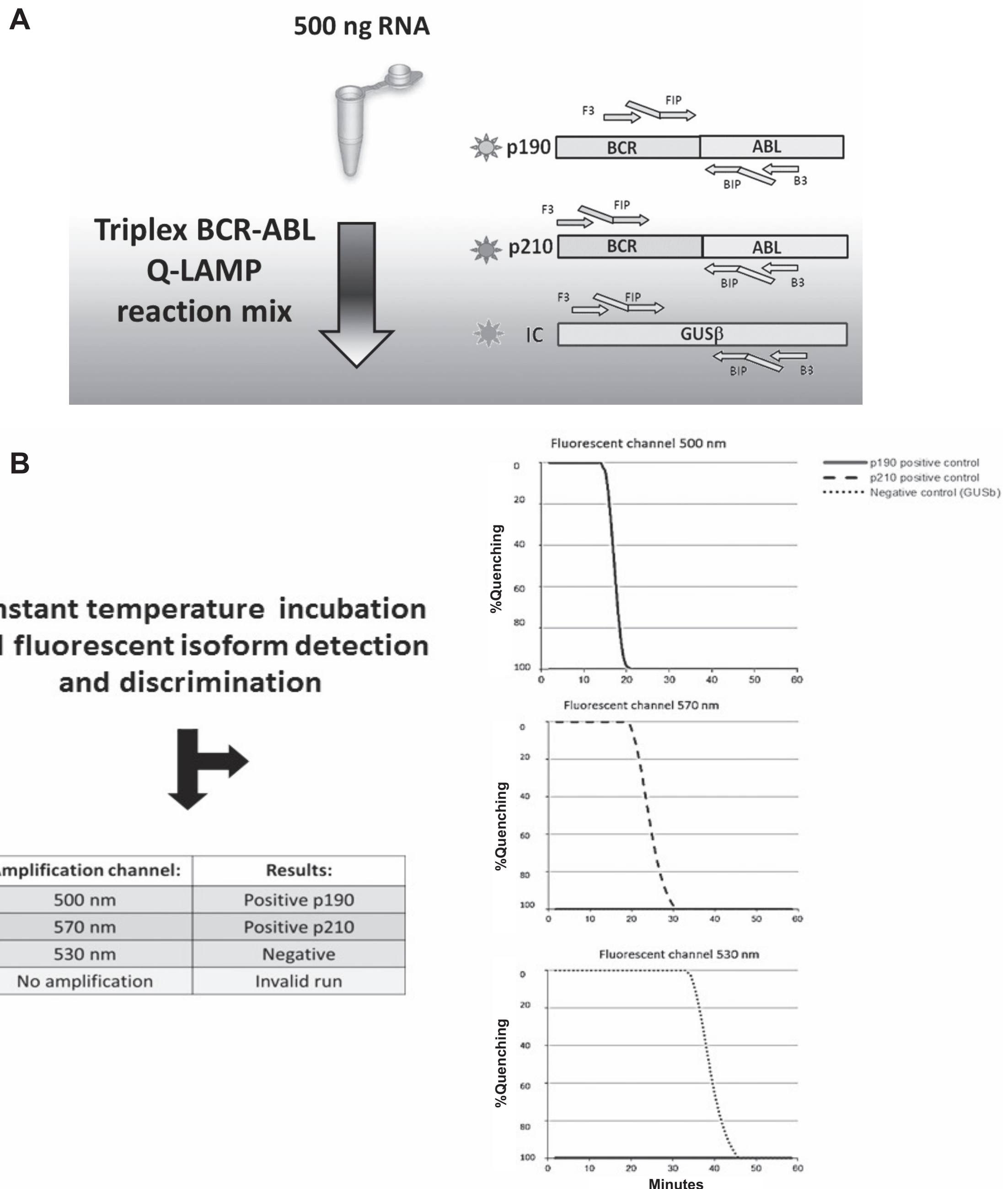
[a]*For the PML-RARA assay, negative sample amplification of the housekeeping gene/internal control is mandatory.*

single tube target amplification with one enzyme at constant temperature. Furthermore, by addition of labeled oligonucleotides, it was possible to monitor the amplification by fluorescence variations in a real-time way onto the Liaison Iam analyzer (DiaSorin). At time zero, the reaction excited with appropriate wavelength UV light emits a maximum fluorescence. As a function of amplification, the fluorescence decreases exponentially thanks to the natural quenching effect of newly synthetized amplicons (Q-LAMP). The threshold time is the minute at which the sample fluorescence reaches the 50% of quenching and is correlated with the amount of target present in the reaction (22). For Q-LAMP assays dried reagents are provided for analysis in an 8 tubes strip format and endogenous *GUSβ* housekeeping transcript is amplified as internal control in the same tube of the target reaction to check extraction procedure, RNA integrity, reaction functionality and absence of inhibitors (Figure 2). To avoid false negative results, the control gene amplification is calibrated to be weak and sensitive to RNA quality: low quality RNA samples do not provide control gene signal, prompting test repetition. Also translocation positive samples present a low control gene signal due to competition for reagents during reactions.

The PML-RARA Q-LAMP assay (Iam PML-RARA kit, DiaSorin S.p.A) consists of two fluorescent multiplex amplifications, one specific for the most frequent transcripts (bcr1 and bcr3) and one for the rarer bcr2 (22). One patient sample can be analyzed for the complete panel of *PML-RARA* transcripts in a single step. For bcr1 and bcr3 detection assay, lyophilized Mix A is dissolved with 60 μL of buffer 1 and 60 μL of buffer 2 and then added to Mix B. The final bcr1, 3 mixture is dispensed on ice in tube 1 to 5 of the strip (20 μL/tube). Then, 5 μL of patient's RNA (100 ng/μL) and supplied controls (bcr1 positive control, bcr3 positive control, negative control, no amplification control) are separately added to the first 5 tubes. For bcr2 discrimination, 50 μL of buffer 3 and 4 are added to Mix C and dispensed in tubes 6 to 8 (20 μL/tube). In the same manner, 5 μL of bcr2 positive control, negative control and patient's RNA are added into the last 3 tubes. The strip is then centrifuged and loaded on the Iam instrument for iso-thermic amplification. Samples positive in the bcr1, 3 assay have then to be checked in the bcr2 assay for transcript discrimination as explained in Table 1.

The Q-LAMP assay for *BCR-ABL1* amplification (Iam BCR-ABL1 kit, DiaSorin S.p.A) detects and discriminates the Minor (p190) and the Major (p210) transcripts in a single tube (Figure 2). Resuspended reaction mixes are dispensed into provided strip tubes and 5 μL of supplied controls (negative control, positive control for p190 and p210 transcripts and no amplification control) and patient's RNA (100 ng/μL) are added in separate tubes. Up to 5 samples can be analyzed in a single experimental section. During amplification, samples positive for p190 generate a signal in 500nm channel, while samples positive for p210 generate a signal in 570 nm channel. Negative samples generate a signal in 530 nm channel deriving from control gene amplification (Figure 2, panel B).

The allele specific (AS)-LAMP is an allele specific reaction based on LAMP principle that we developed and described for JAK2 V617F identification (19). The amplification proceeding is followed in a real time manner by a turbidimeter measuring the production of pyrophosphate precipitate, a byproduct of nucleotides incorporation during DNA synthesis (19, 23). This assay



Amplification channel:	Results:
500 nm	Positive p190
570 nm	Positive p210
530 nm	Negative
No amplification	Invalid run

Figure 2
BCR-ABL1 Q-LAMP assay.
The BCR-ABL1 Q-LAMP assay is characterized by a triplex fluorescent test in which p190, p210 isoforms and GUSβ control gene are amplified in a single tube at isothermal condition (panel A). The triplex fluorescent BCR-ABL1 test amplifies p190 positive samples in the 500 nm channel, p210 positive samples in the 570 nm channel, while the GUSβ control gene in the 530 nm (panel B).

contains a peptide nucleic acid (PNA) that blocks the wild type allele amplification and a self-annealed primer that enhances the mutated allele amplification (Figure 3). To validate negative results, a parallel reaction should be performed in a separate tube with the same primer set omitting PNA and self-annealed primer so allowing the mutant-insensitive amplification of the *JAK2* gene. To obtain a one-tube reaction we modified this first assay version by adding an intercalating dye (Yo-Pro-1 iodide, Molecular Probes, Invitrogen) as well as an additional primer set specifically designed to amplify the endogenous *ABL1* gene as an internal control. The reaction is performed at 65 °C for 30 min on 25 ng of genomic DNA, in a total of 25 µL of reaction mixture, in a real time Optical thermoblock (GenieI, Optigene), able to incubate at constant temperature, to detect the emitted fluorescence level and to perform annealing analysis on the amplified products. The level of

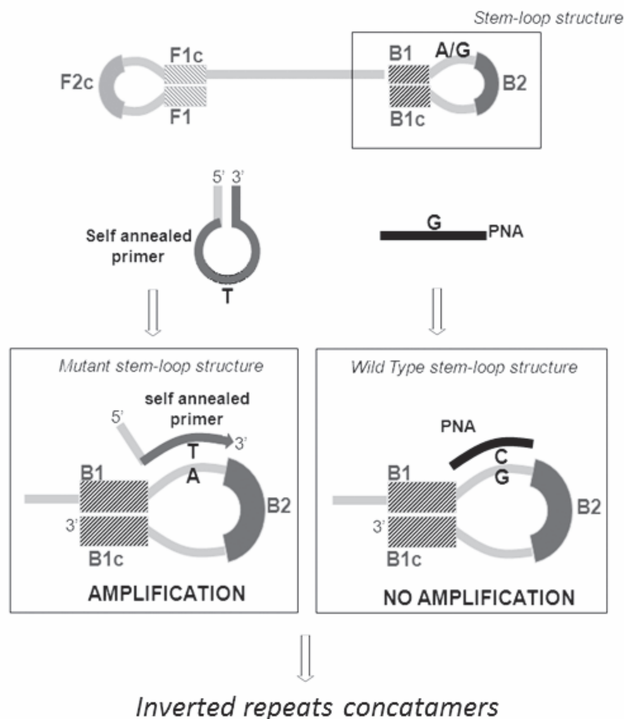


Figure 3
Allele specific (AS)-LAMP method.
The target DNA is extended by FIP and BIP primers and the newly synthesized DNA chains are displaced by extension of F3 and B3 generating a stem-loop structure. The displaced product represents the starting structure of LAMP reaction. In the presence of JAK2 wilt-type (WT) sequence a specific peptide nucleic acid (PNA) forms a stable duplex with the stem-loop structure, which cannot be extended by the polymerase. On the contrary, in the presence of a mutant JAK2 sequence, the PNA does not anneal, while a self-annealed primer breaks its internal structure to bind the mutated JAK2 target and the LAMP reaction proceeds with the generation of repeats concatamers. To validate negative results a parallel reaction is performed in a separate tube with the same primer set omitting PNA and self-annealed primer, so allowing the mutant-insensitive amplification of the JAK2 gene.

fluorescence increases significantly when the intercalating dye binds the double-stranded DNA during the hybridization and extension step. The fluorescence signal increases proportionally to the amount of amplified product. An annealing analysis is performed at the end of isothermal incubation to discriminate the JAK2 V617F and the *ABL1* products of amplification. The annealing curves were generated by the denaturation at 100 °C of the LAMP product(s) followed by slowly cooling the amplified DNA from 95 °C to 70 °C. The JAK2 V617F and *ABL1* products were characterized by different annealing temperatures (83.7±0.3 °C and 91.2±0.7 °C, respectively) revealed as annealing temperatures peaks [obtained by calculating the negative first derivatives (-dF/dT) of the annealing curve raw data]. Sensitivity of the fluorescent AS-LAMP was evaluated on serial dilution of UKE-1 cell line JAK2 V617F positive DNA into B-JAB cell line wild type DNA. Sensitivity was defined as previously described (24, 25).

RESULTS

Fluorescent Q-LAMP assays for *PML-RARA*

Two Q-LAMP assays were designed to detect the 3 *PML-RARA* chimeric transcripts (bcr1, bcr2 and bcr3). One assay was developed for the simultaneous amplification in a single tube of bcr1, bcr3 *PML-RARA* transcripts and *GUSβ* housekeeping gene as an internal control (triplex assay). This assay may provide molecular diagnosis in 95% of APL cases. A separate assay was set up for the bcr2 *PML-RARA* transcript again including the *GUSβ* amplification (duplex assay). In these assays the amplification of targets can be monitored following the fluorescence variation within the reaction tubes settled into the Liaison Iam instrument, an 8-well manageable instrument suitable for isothermal reactions. This instrument, thanks to the presence of 3 detection channels of fluorescence, can monitor multiplex assays, returning elaborated final objective results with no need of data analysis by the operator. Figure 4 shows representative fluorescence quenching curves for bcr1, bcr3 and *GUSβ* amplifications (panel A, B and C) and for bcr2 and *GUSβ* assay amplifications (panel D and E). These assays were tested for specificity on hundreds of replicates of RNA derived from 8 cell lines negative for *PML-RARA* transcripts as well as on healthy donors derived RNA. All the above samples turned out negative in the assay evaluation. Finally, all the RNA derived from the bcr1 positive NB4 cell line and from bcr2 and bcr3 positive APL patients resulted positive in Q-LAMP assays amplification. Furthermore, Q-LAMP was more efficient in isoform definition when tested on difficult historical cases resolved with Sanger sequencing (22). Finally, 100% concordance between classical RT-PCR evaluation and Q-LAMP evaluation was obtained when the two methods were used in parallel into the diagnostic process of incoming patients with a suspicions of APL (n=17). The average detection time of *PML-RARA* transcripts with Q-LAMP was 16±1 min, while *PML-RARA* negativity (amplification of *GUSβ* only) could be assessed in non APL samples with an average threshold time of 30 min. Thus, PML-RARA Q-LAMP assays provide molecular diagnosis of APL at least 4 h before RT-PCR. Sensitivity of the Q-LAMP was evaluated on serial positive RNA 10-fold dilutions in wild type HL-60 RNA (negative for *PML-RARA* translocation). Bcr1 and bcr3 assays reached a 10^{-3} sensitivity, whereas bcr2 assay sensitivity was 10^{-2}.

Fluorescent Q-LAMP assay for *BCR-ABL1*

Thanks to the employment of fluorescent specific oligonucleotides, *BCR-ABL1* transcripts detection was set up as multiplex Q-LAMP in a single tube. The Minor (p190) and Major (p210) t(9;22) transcripts are retro-transcribed and amplified with the endogenous GUSβ

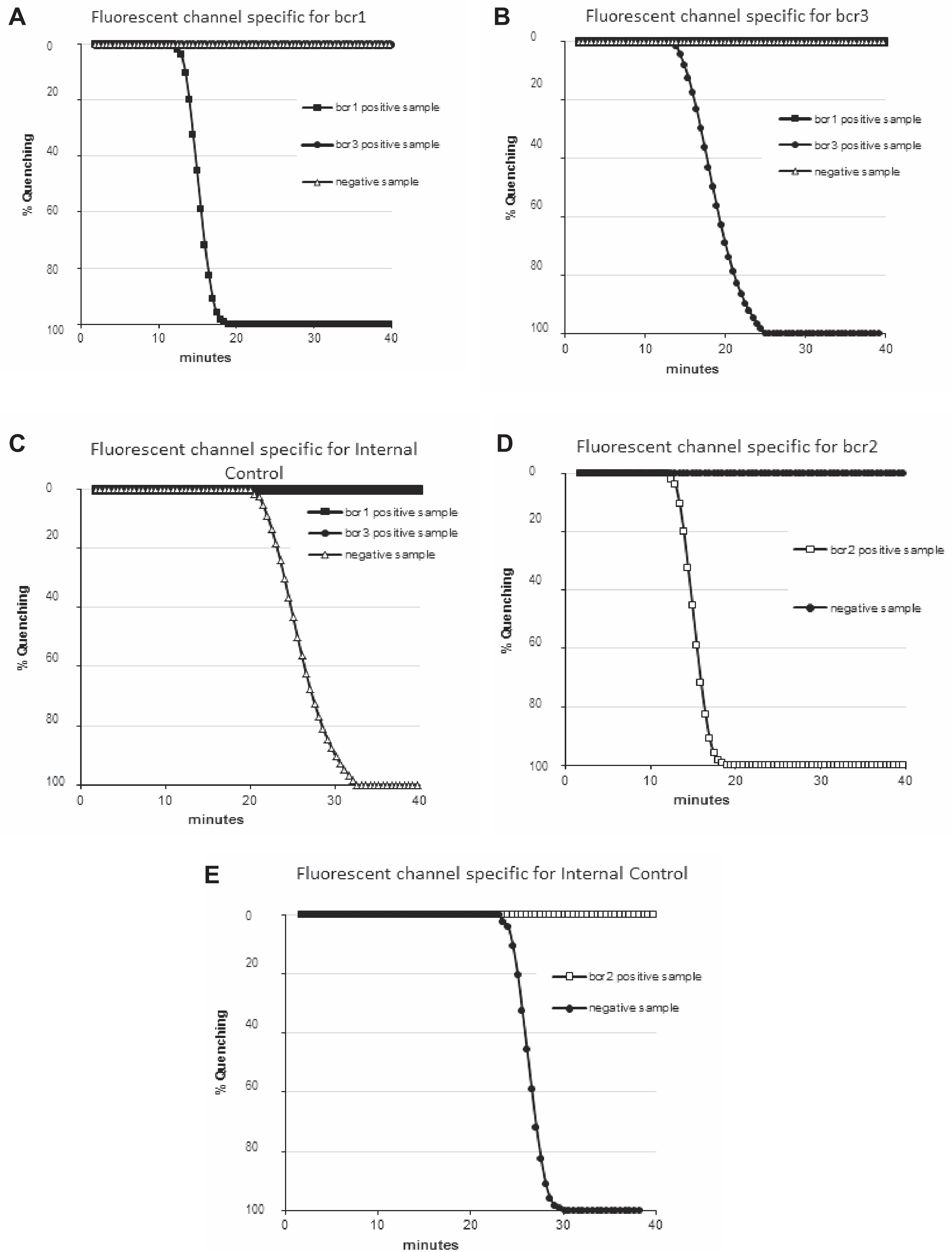


Figure 4
PML-RARA Q-LAMP amplification plots. Amplifications curves of bcr1 (panel A), bcr3 (panel B) and bcr2 (Panel D) in PML-RARA positive samples obtained by triplex and duplex assays are represented. Negative samples, in which only GUSβ gene is amplified, are also shown (panel C and E).

RNA used as internal control for validation of negative results. Q-LAMP is carried out on the Liaison Iam at constant temperature. Q-LAMP assay sensitivity was evaluated on serial dilutions of RNA derived from positive cell lines (TOM-1 or K-562 for p190 and p210, respectively) into RNA from HL-60 cell line, negative for *BCR-ABL1* transcripts (30 replicates). The p190 and p210 transcripts were detected and distinguished down to 10^{-4} and 10^{-5}, respectively, within 50 min (Figure 5). The assay demonstrated 100% specificity since 70 replicates of wild type RNA from 7 cell lines turned out *BCR-ABL1* negative and *GUSβ* positive (internal amplification control). This assay was also validated on 60 clinical samples (30 positive for p210, 30 positive for p190 transcript) obtained at disease diagnosis, all previously analysed by conventional RT-PCR. Q-LAMP detected and identified the *BCR-ABL1* fusion transcripts correctly, presenting a 100% concordance with the reference RT-PCR method. Fully concordant results were obtained also on 30 RNA samples from patients affected by Philadelphia-negative diseases and on 30 RNA samples, obtained from healthy donors: in all these samples, Q-LAMP amplified only the housekeeping *GUSβ* transcript as expected. Similarly to *PML-RARA*, also *BCR-ABL1* transcripts amplification showed a quantitative pattern when serially diluted in negative RNA (Figure 5), suggesting the possibility to further implement the assay toward a quantitative format.

Fluorescent AS-LAMP assay for JAK2 V617F mutation identification

The AS-LAMP assays for JAK2 V617F mutation identification was initially developed as a non-fluorescent method (19). We then implemented the AS-LAMP by the addition of an intercalating fluorescent dye to allow the amplification of the *ABL1* housekeeping gene in the same tube with specific oligonucleotides. To distinguish the specific product and to avoid false positive results due to non-specific amplification like primer-dimers, an annealing analysis was performed at the end of isothermal incubation to discriminate the JAK2 V617F and internal control *ABL1* products. JAK2 V617F and *ABL1* were identified by different annealing temperatures and visualised with an annealing curve (Figure 6). The specificity of the AS-LAMP assay was proved performing the reaction on 135 replicates of genomic DNA extracted from 5 cell lines negative for JAK2 V617F mutation (REH, RS411, MV4, 697, HL60). One positive control from the UKE-1 cell line (carrying the V617F mutation) and a No Target Control (NTC, nuclease-free water) sample were included in the experimental procedure. The *ABL1* gene was correctly amplified in all the tested samples, generating the expected annealing peak at 91.2±0.7 °C. No amplification occurred in NTC, while positive UKE-1 cell line produced an amplicon with an annealing temperature specific for JAK2 V617F mutation (84 °C) (Figure 6, panel A). Therefore, the method demonstrated a level of specificity of 100% on a high number of replicates. The sensitivity of the fluorescent duplex AS-LAMP assay was assessed on genomic DNA from UKE-1 cell line serially diluted into B-JAB DNA (negative for JAK2 mutation). The dilutions were prepared to obtain a percentage of mutant JAK2 allele in wild type allele of 10%, 1%, 0.5%, 0.1%, 0.05% and 0.01%. The JAK2 V617F mutation was detected within 30 min down to 0.05% of mutated DNA in wild type, which represents the maximum sensitivity of the assay (detected in 49% of cases on 65 replicates). The reproducible sensitivity (25) is one log less, since the

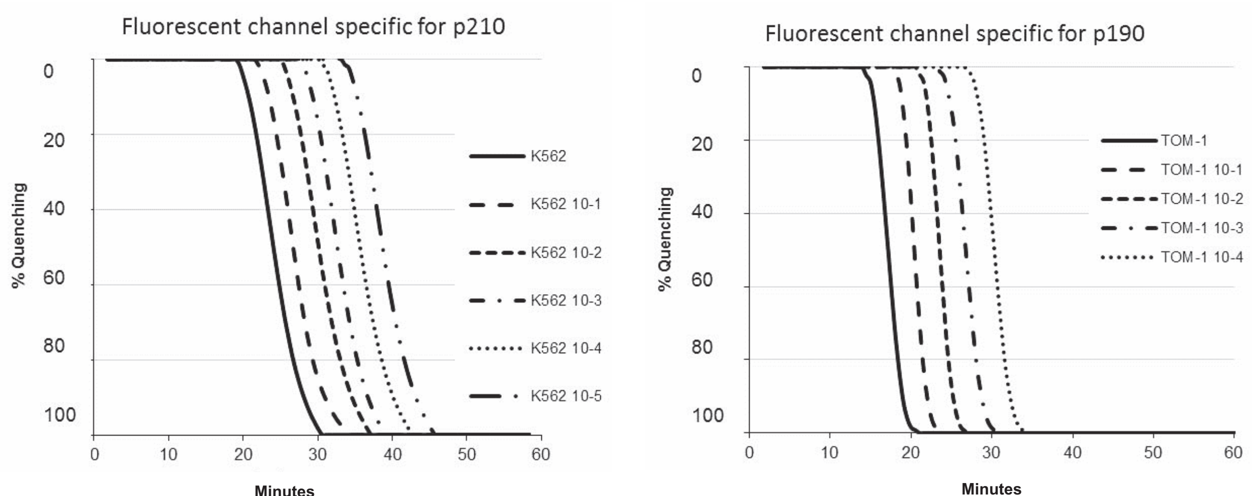


Figure 5
Sensitivity of BCR-ABL1 Q-LAMP assay.
The sensitivity of BCR-ABL1 Q-LAMP was tested on serial dilutions in HL60 RNA of K562 RNA for p210 isoform (panel A) and TOM -1 RNA for p190 isoform (panel B). The experimental sensitivities were 10^{-5} and 10^{-4} for BCR-ABL1 p210 and p190 isoform, respectively.

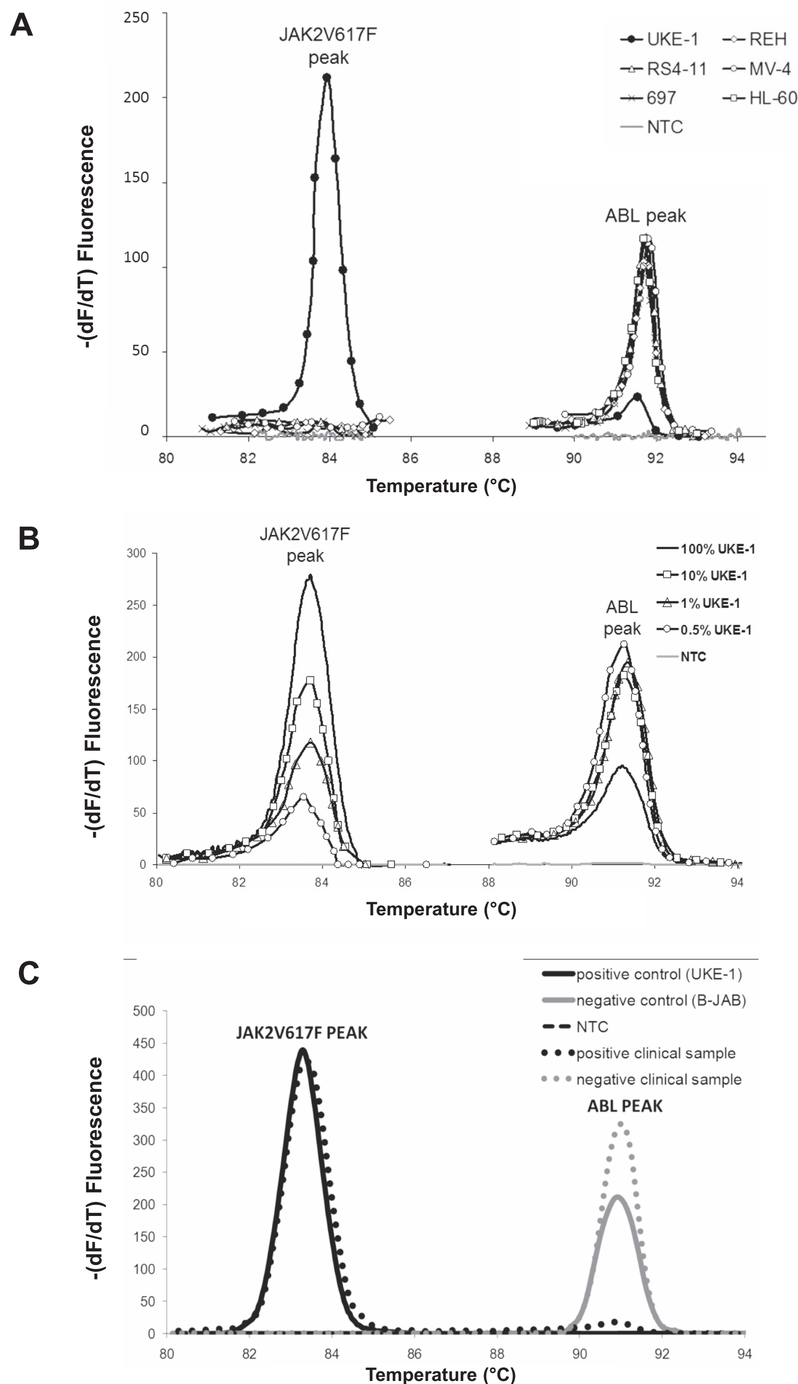


Figure 6

Specificity and sensitivity of allele specific (AS)-LAMP assay.

The fluorescent duplex AS-LAMP assay was performed on 5 wild-type (REH, RS411, MV4, 697, HL60) and one JAK2 V617F mutant cell lines (UKE-1). The method demonstrated a specificity of 100% on all replicates. The amplification product of UKE-1 cell line showed an annealing temperature specific for JAK2 V617F mutation at 84 °C. No amplification occurred in REH, RS411, MV4, 697, HL60 cell lines and NTC (No Target Control) samples. The ABL1 gene was correctly amplified in all tested DNA, with an annealing temperature of 91.2±0.7 °C (panel A). The sensitivity of the fluorescent duplex AS-LAMP assay was assessed on undiluted UKE-1 mutant DNA (100%) and on serial dilutions of UKE-1 mutant DNA into wild type DNA from B-JAB cell line at concentrations of 10%, 1% and 0.5%. All the samples showed a peak at the annealing temperature of the amplification product ABL1 (internal control) and a peak at the annealing temperature of the JAK2 V617F product. Moreover, the height of the JAK2 V617F peak was proportional to the amount of starting mutant target. The NTC sample was not amplified (panel B). A fluorescent duplex AS-LAMP on clinical samples is also presented (panel C). The positive clinical sample showed a peak at the specific annealing temperature for JAK2 V617F and a very weak peak in correspondence to ABL1 annealing temperature due to the competition for reagents of the two reactions. Negative clinical sample produced only one peak at the ABL1 control gene annealing temperature.

0.5% dose has been detected in 100% of cases on a high number of replicates (n=103). Interestingly, a direct relationship between the amount of mutant target tested and the height of peaks obtained after annealing analysis has been consistently observed (Figure 6, panel B), suggesting a possible further development of a fully quantitative assay. The fluorescent duplex AS-LAMP has been validated on 27 JAK2 V617F mutated clinical samples derived from patients routinely followed at Hematology and Bone Marrow Transplant Unit of Papa Giovanni XXIII Hospital (Figure 6, panel C). The AS-LAMP data were perfectly concordant with the ones obtained by conventional ASO-PCR. The high sensitivity level of the assay allowed to correctly detect the JAK2 V617F mutation on selected granulocytes as well as on DNA samples extracted directly from whole peripheral blood, avoiding the step of granulocytes isolation. In addition, we tested 19 clinical specimens previously resulted negative by conventional ASO-PCR, including 10 DNA samples obtained from granulocyte of healthy donors and 9 DNA samples obtained from blood of patients with different hematologic disorders [two acute lymphoblastic leukemia, two follicular non-Hodgkin's lymphoma, two B cell chronic lymphocytic leukemia (B-CLL) and 3 ET]. We found only one sample not in agreement: one ET patient previously found negative by ASO-PCR was found clearly positive with duplex AS-LAMP. A further investigation demonstrated that this patient was positive for the V617F mutation in *JAK2* gene at a very low level. Indeed, a DNA specimen obtained one month later was confirmed positive by AS-LAMP and low positive also by ASO-PCR method. Possibly, the positivity of the first sample was not detected due to the lower sensitivity of conventional method. After this validation, the AS-LAMP was also employed on a cohort of 158 B-CLL patients (26). This approach led us to detect the JAK2 V617F mutation in 4 patients affected by this lymphoproliferative disease (~2.5%), with a low allele burden (~0.5%). Interestingly, the concomitant presence of this molecular lesion in B-CLL patients has been reported by another group of investigators (27).

DISCUSSION

In this study, we have described new LAMP-based assays for the molecular diagnosis of hematologic malignancies. We modified the original LAMP approach by introducing a fluorescence detection system and a polymerase that can work directly on RNA and specific molecular elements for single nucleotide variation determination. These modifications allowed amplifying in a single tube and in a single step different targets and control genes. These characteristics eliminate multiple manipulations that in classical PCR represent possible contamination source and take considerable operator and machine time. Moreover, the number of tubes to be managed for a single patient is reduced as well as the amount of nucleic acid required that in some cases can be a limiting factor for a proper diagnosis. The possibility to discriminate the different isoforms by the Q-LAMP test is also important for the subsequent molecular monitoring by the gold standard RQ-PCR, usually performed to verify treatment efficacy and to modulate drugs administration (14). The amplification process can be visualized on the instrument during the reaction proceeding allowing the identification of positive samples within 15-30 min, before run conclusion. The rapidity of the assay can be of high value in some peculiar circumstances, such as the diagnosis of APL, in which starting as soon as possible the appropriate life-saving treatment based on ATRA is crucial. Similarly to APL, CML and Philadelphia-positive ALL have a dramatic benefit from a targeted therapy with TKI (4-8). Although in the latter cases there is no need to retrieve this information in a few hours, it is true that worldwide the need of an accurate diagnostic work up to detect this crucial genetic lesion is far from being appropriately met.

BCR-ABL1 Q-LAMP could be important also in the MPN work-up. In fact, the diagnostic process in Philadelphia-negative MPN requires the exclusion of the t(9;22) translocation. The absence of this molecular lesion can easily redirect the molecular analysis toward the JAK2 V617F mutation, which represents a hallmark of most MPN. In this context, fluorescent AS-LAMP technology can offer a rapid and efficient JAK2 V617F detection also at low levels. In case of a confirmed MPN negative for JAK2 V617F, further molecular analysis can be promptly performed on other commonly mutated targets (JAK2 exon 12 for PV, CALR exon 9 or MPL exon 10 for ET and MF) (28).

It is important to realize that the hematologic molecular diagnosis is often performed by specialized laboratories and in some countries this kind of diagnosis is centralized in one or few laboratories that can be far from the clinic where the patient firstly refers to. This organization process raises the problem related to sample shipment (delivery time and transport conditions, such as temperature and safety) and delays molecular diagnosis. The one-step assay configuration and the automatic result interpretation make LAMP-based assays feasible also in not specialized laboratories managing few samples per year. Therefore, an immediate molecular diagnosis can be performed and patient can be promptly referred to a specialized center for the cure of the specific neoplasia in a short time. On the other hand, the possibility of increment the instrument output to 48 samples per run through serial instruments configuration can make these assays suitable also for specialized laboratories. In these centers, the high number of clinical samples and molecular markers to be sequentially studied for each disease following international recommendations requires rapid, sensitive and reliable techniques. All in all, the described assays are fast, simple, specific, robust and reliable and can be adopted by laboratories with different organization and expertise. Further improvements as automation of sample processing, nucleic acid extraction and amplification set up are in

progress to optimize LAMP-based assay workflow and make this technology even more attractive.

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CONFLICTS OF INTEREST

Alessandro Rambaldi e Orietta Spinelli are consultant of DiaSorin.

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